Tropical Medicine, 24(3), 155–163, September, 1982

Acceleration of Polymerization of ADP-ribosylated Tubulin by Cholera Toxin

Masahiko EHARA* and Hideaki SHIGENO**

Department of Bacteriology, Institute for Tropical Medicine, Nagasaki University, 12-4 Sakamoto-cho, Nagasaki 852, Japan.

Abstract: We have examined the effect of cholera toxin on microtubule assembly in the presence of the nucleotide regenerating system. Our results indicate that ADP-ribosylated tubulin increases its stability and accelerates its polymerization forming microtubules decorated with rings of 46nm in diameter.

INTRODUCTION

Cholera toxin exerts its effect on cells through ADP-ribosylation of a GTP-binding protein, which is a regulatory component of adenylate cyclase[1)~6)]. *In vitro* cholera toxin catalyzes the NAD to ADP-ribose and nicotinamide[7)]. It is obvious that the A_1 peptide of the toxin catalyzes the NAD-dependent ADP-ribosylation of constitutional proteins in cell that have no relation to the adenylate cyclase system[8)]. Hebdon *et al.*[9)] noted that A_1 peptide modified $3T3C_2$ mouse fibroblast membrane proteins of 45,000 daltons and of a doublet of polypeptides around 52,000 daltons. Johnson *et al.* observed the cholera toxin-and NAD-dependent ADP-ribosylation of membrane proteins of 45,000 and a doublet of 52,000 to 53,000 daltons in wild type S49 mouse lymphoma cells. These results suggest that the A_1 peptide plays another function in cells via ADP-ribosylation of cell components. Although tubulin may comprise as much as 40% of the soluble protein of the brain[10)11)], there have been several observations suggesting that tubulin is also associated with the plasma membrane. Analyses of proteins in membrane fractions have provided evidences for the presence of tubulin in synaptic membranes[12)~14)], microsomal membranes[15)], brain and thyroid membranes[16)], and flagellar and cilia membranes[17)]. Estridge[18)] and Zisapel[13)] suggested that tubulin could be an integral membrane protein in the axonal plasma membranes and mammalian synaptic vesicle membranes. Recently Kaslow *et al.* reported that cholera toxin could catalyze ADP-ribosylation of α, β-

* To whom correspondence should be addressed.
** Present address: Department of the 2nd Internal Medicine, Nagasaki University Hospital.
Received for publication, September 21, 1982.
Contribution No. 1248 from the Institute for Tropical Medicine, Nagasaki University.
Abbreviations: DTT, dithiothreitol; MES, 2-(N-morpholino) ethene sulfonic acid; ECTA, ethyleneglycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid; SDS, sodium dodebyl sulfate.

tubulin[19]. It is interested to note what functional consequences may result from ADP-ribosylation of α, β-tubulin by cholera toxin. From the results mentioned above we doubt that cholera toxin exerts its biological effect on cells through the activated adenylate cyclase system alone. Thereby, ADP-ribosylation of cytoskeletal proteins and plasma membrane proteins might play some important roles for cells. These urged us to investigate the ADP-ribosylation of tubulin in detail. In this communication we deal that the polymerization of tubulin is accelerated by cholera toxin via ADP-ribosylation.

MATERIALS AND METHODS

Materials: Reagent-grade chemicals were purchased from the following sources: GTP, DTT, 2-mercaptethanol, and bovine serum albumin from Sigma Chemical Co., MES, EGTA, from Nakarai Chemicals Ltd., Cholera toxin from The Chemo-Sero-Therapeutic Research Institute, NAD, creatine-kinase from Boehringer Manheim GmbH.

Buffer: The modified reassembly buffer (MRB) of Weisenberg[20] composed of 0.1M MES containing 1mM EGTA, 0.5mM GTP, and 0.5mM $MgCl_2$(pH 6.8) were used.

Methods: Porcine brain tubulin was purified by temperature-dependent cycles of assembly-disassembly as described by Shelanski *et al.* with some modifications. After two cycles of polymerization, tubulin pellet was dipersed into MRB without glycerol and incubated for 30 min at 35°C. The mixture was centrifuged at 100,000×g for 1hr at 25°C, and the pellet again dispersed into cold MRB, followed by recentrifugation at 100,000×g for 40 min at 4°C. The supernatant obtained was divided into small portions and stored at −80°C untill use. ADP-ribosylation of tubulin was assayed by incubating the purified protein at 1mg/ml and 35°C in MRB with 0.1mM NAD, 50μg/ml DTT-treated cholera toxin[22], and nucleotide triphosphate regenerating system consisting of 20mM creatine phosphate and 0.1mg/ml creatine phosphokinase[23]. Polymerization was initiated by adding 0.5mM GTP and followed by warming to 35°C in a thermostatically controlled cuvette chamber of a Shimadzu UV-240 spectrophotometer. The increase in turbidity was monitored at 350nm. When polymerization of the mixture had reached a plateau, one drop of sample for electron microscopy was placed on a Formvar-carbon-coated grid. All samples were stained negatively with 1% uranyl acetate for 30s, and were washed three times with distilled water. The excess water was drained off with filter paper. Electron microscopy was performed in a JEM 100 CX at 70kV.

Gel electrophoresis: SDS-polyacrylamide gel electrophoresis was performed on 9% slab gel according to the method of Laemli[24] to monitor the purification of tubulin. Protein was determined by the method of Lowry *et al.*[25] with bovine serum albumin as a standard.

RESULTS

Tubulin purified by 3 cycles of polymerization-depolymerization showed only two

bands indicating α, β-tubulin subunits in a SDS-gel (data not shown). Effects of cholera toxin on polymerization: Spectrophotometrical observation; Fig. 1 shows an increase of turbidity due to microtubule assembly under two conditions. ADP-ribosylation of tubulin in the presence of nucleotide regenerating system caused the turbidity to increase slightly compared with that in MRB alone. Electron microscopic observation; Assembly in MRB was compared to assembly of similar samples in 4M glycerol or after ADP-ribosylation by DTT-treated cholera toxin. At low magnification, polymerization is accelerated by ADP-ribosylation compared with the sample in MRB alone or 4M glycerol (Figs. 2a-c). At high magnification, microtubules reassembled in MRB alone show twisted forms, but are of normal morphology (Fig. 3a). However in the sample reassembled in 4M glycerol (Fig. 3b), normal microtubules and another forms unrolled in a sheet are also observed as reported by Shelanski[20]. ADP-ribosylated sample shows a few microtubules of fine structure and numerous alternate forms decorated with rings of 46nm in diameter (Fig. 3c). These alternate forms are distinct from those unrolled in a sheet (Fig. 3b) in their width.

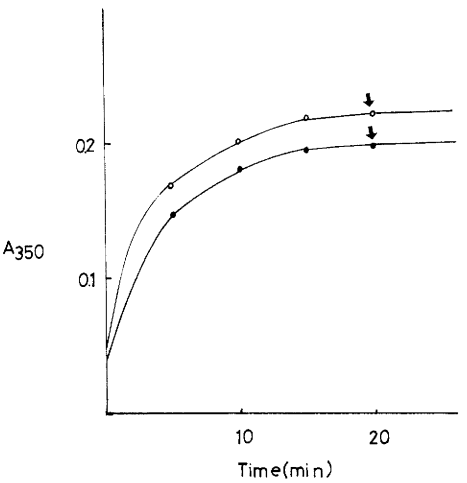

Fig. 1. Effect of ADP-ribosylation on microtubule assembly.
Purified-Shelanski-tubulin (1mg/ml) was dissolved in MRB. Changes in turbidity of the tubulin solution were measured at 35°C after the addition of 0.5mM GTP (●), and 0.5mM GTP containing 0.1mM NAD, 50μg/ml DTT treated cholera toxin and 20 mM creatine phosphokinase (○). Arrows indicate the time when samples for electron microscopy were taken.

DISCUSSION

Tubulin seems to be existed not only in cytoplasm and nucleoplasm, but also in membrane fractions in eukaryotic cells. The large variety of functions, together with transitory assembly of many microtubules, implies the existence of regulatory signals which modulate the polymerization of tubulin during differentiation, cell division, and cell movement as well as neurotransmission. Understanding the mechanism and control of microtubule assembly has been facilitated by the development of conditions for the polymerization *in vitro* and chemical modification of tubulin. Nath and Flavin showed tubulin tyrosylation *in vivo* accompanying differentiation of cultured neuroblastoma glioma hybrid cells[26]. Vigny *et al.* reported that tyrosine hydroxylase was able to in-

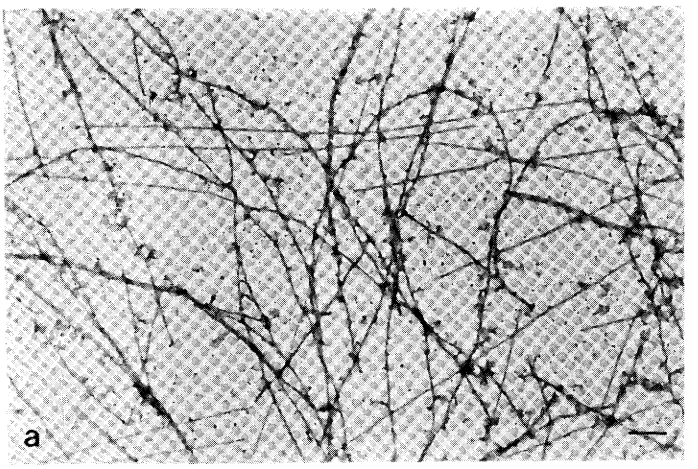

Figure 2-a

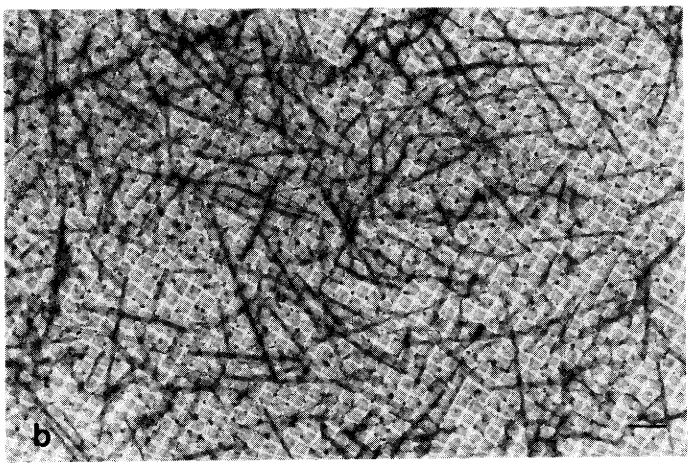

Figure 2-b

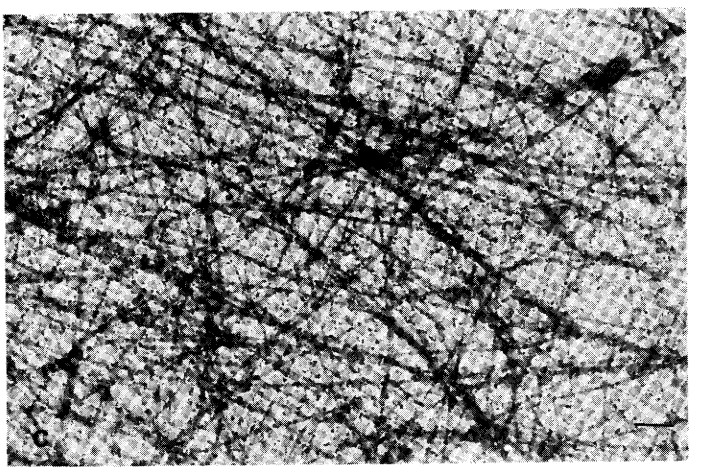

Figure 2-c

Fig. 2. Electron microscopic comparison of microtubules reassmbled in MRB alone (a), 4M glycerol (b) and ADP-ribosylating system (c). Negative staining. Bars indicate 0.5μm. Magnification: $\times$10,500.

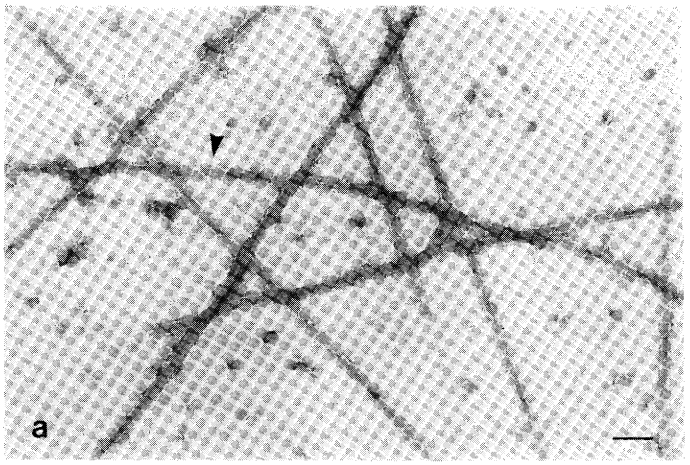

Figure 3-a

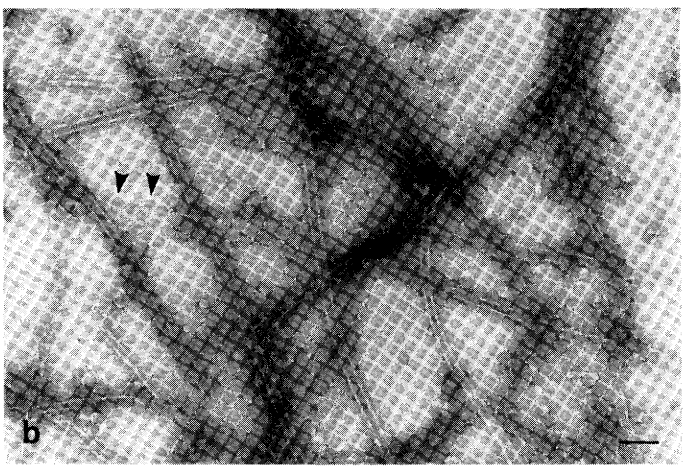

Figure 3-b

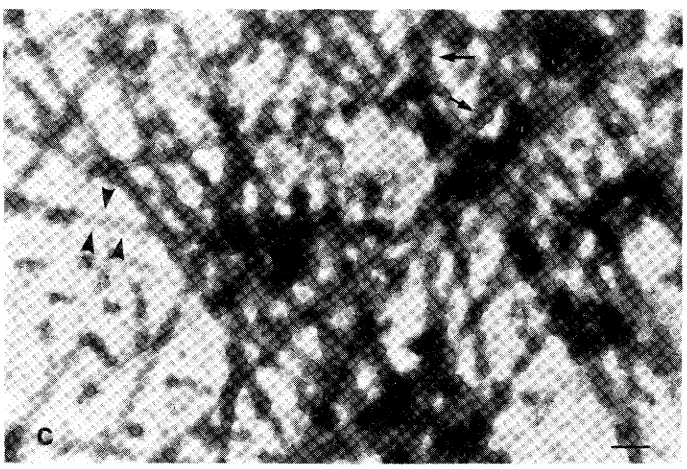

Figure 3-c

Fig. 3. Enlarged selected images of reassembled microtubules in MRB alone (a), 4M glycerol (b), and ADP-ribosylating system (c). Arrowheads indicate microtubules unrolled in a sheet. Arrows (Fig. 3c) indicate double-walled rings of 46nm in diameter. Bars indicate 0.1μm. Magnification: $\times$52,000.

duce purified tubulin assembly like the microbubule associated protein [27]. But in this system, the association induced by tyrosine hydroxylase resulted in the formation of aggregates or organized structure different from microtubules. Hayashi *et al.*[28] and Shimo-Oka *et al.*[29] reported that the evidences suggested the direct binding between tubulin and myosin showed the different ability of tubulin from that to form microtubules. Feit and Shay[30] reported that bovine brain tubulin was ressembled into an alternate form that resembled membranes rather than microtubules.

On the other hand, cholera toxin has been used to raise the level of intracellular cyclic AMP. The possible role of cyclic AMP in the regulation of cell division is a subject of much contraversy. Hollenberg *et al.* reported that cholera toxin inhibited DNA synthesis of human fibroblasts and transformed mouse epithelial cells[31][32]. Similar inhibitory effects have been observed with lymphocytes of mice and humans[33]~[35]. In contrast to these observations, treatment with cholera toxin and other procedures to increase the cyclic AMP level were reported to stimulate DNA synthesis and cell growth. Such stimulatory effects of cholera toxin have been shown with several types of cells including melanoma cells[36], Schwann cells[37], 3T3 cells[22], human mammary epithelial cells[38], and human microvascular endothelial cells[39]. Green also reported that colonial growth of human epidermal cells was enhanced by cholera toxin[40]. Kuroki confirmed the result of Green [41]. Is cyclic AMP a signal for DNA synthesis ?

We propose that ADP-ribosylation of tubulin in nucleoplasm may be a signal for DNA synthesis triggering tubulin polymerization in eukaryotic cells. Our data indicate that ADP-ribosylation of tubulin induces many ring forms as observed in electron micrograph (Fig. 3c) and promotes tubulin polymerization. Zabrecky and Cole also reported that ATP induced aggregates of double-walled rings (46nm diameter). Some problems are left to be settled down. Where is the binding site of ADP-ribose to tubulin ? One diamer of α,β-tubulin has two GTP binding sites as named N site and E site. But according to the data by Kaslow *et al.*[19], each of α and β-tubulin has been ADP-ribosylated. There may be another a third ADP-ribose binding site. Dose ADP-ribosylation of tubulin inhibit the GTPase activity just as dose the GTP binding protein in adenylate cyclase system ? We used the porcine brain tubulin as the sample for ADP-ribosylation. It may be likely that the integrity of epithelial cell membranes in intestinal tract is disturbed by ADP-ribosylation of tubulin in plasma membrane resulting watery diarrhea.

ACKNOWLEDGEMENTS

We thank Dr. R. A. Finkelstein and Dr. Joel Moss for useful discussions at the International Symposium on Bacterial Diarrheal Diseases at Osaka in Japan. We are grateful to Dr. N. Ohtomo for his kind advice in our experiment and to Dr. E. Nishida for his support in the tubulin preparation. The authors wish to express special appreci-

ation to Dr. M. Koike for his helpful criticism and suggestions in the preparation of the manuscript.

REFERENCES

1) Gill, D. M. & King, C. A. (1975): The mechanism of action of cholera toxin in pigeon erythrocyte lysates. J. Biol. Chem., 250, 6424−6432.
2) Cassel, D. & Pfeuffer, T. (1978): Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. Proc. Natl. Acad. Sci. U. S. A., 75, 2669−2673.
3) Gill, D. M. & Meren, R. (1978): ADP-ribosylation of membrane proteins clataized by cholera toxin: Basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U. S. A., 75, 3050−3054.
4) Johnson, G. L., Kaslow, H. R. & Bourne, H. R. (1978): Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. J. Biol. Chem., 253, 7120−7123.
5) Moss, J. & Vaughan, M. (1979): Activation of adenylate cyclase by choleragen. Ann. Rev. Biochem., 48, 581−600.
6) Moss, J. & Vaughan, M. (1981): Mechanism of action of choleragen and E. coli heatlabile enterotoxin: activation of adenylate cyclase by ADP-ribosylation. Mol. Cel. Biochem., 37, 75−90.
7) Moss, J., Manganiello, V. C. & Vaughan, M. (1976): Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A promoter: Possible role in the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U. S. A., 73, 4424−4427.
8) Moss, J. & Vaughan, M. (1978): Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. Proc. Natl. Acad. Sci. U. S. A., 75, 3621−3624.
9) Hebdon, G. M., Le Vine Ⅲ, H., Sahyoun, N. E., Schmitges, C. J. & Cuatrecasas, P. (1980): Demonstration of choleragen-dependent ADP-ribosylation in whole cells and correlation with the activation of adenylate cyclase. Life Sci., 26, 1385−1396.
10) Dutton, G. R. & Barondes, S. (1969): Microtubular protein: Synthesis and metabolism in developing brain. Science, 166, 1637−1638.
11) Bamburg, J. R., Shooter, E. M., & Wilson, L. (1973): Developmental changes in microtubule protein of chick brain. Biochem., 12, 1476−1482.
12) Feit, H. & Barondes, S. (1970): Colchicine-binding activity in particulate fractions of mouse brain. J. Neurochem., 17, 1355−1364.
13) Zisapel, N., Levi, M. & Gozes, I. (1980): Tubulin: An integral protein of mammary synaptic vesicle membrane. J. Neurochem., 34, 26−32.
14) Strocci, P., Brown, B. A., Young, J. D., Bonventre, J. A. & Gilbert, J. M. (1981): The characterization of tubulin in CNS membrane fractions. J. Neurochem., 37(5), 1295−1307.
15) Soifer, D. & Czosnek, H. (1980): Association of newly synthesized tubulin with brain microsomal membranes. J. Neurochem., 35, 1128−1136.
16) Bhattacharyya, B. & Wolff, J. (1975): Membrane-bound tubulin in brain and thyroid tissue.

J. Biol. Chem., 250, 7639−7646.

17) Stephens, R. E. (1977): Major membrane protein differences in cilia and flagella: Evidence for a membrane-associated tubulin. Biochem., 16, 2047−2058.

18) Estridge, M. 1977. Polypeptides similar to the -and -subunits of kubulin are exposed on the neuronal surface. Nature, 268, 60−63.

19) Kaslow, H. R., Groppi, V. E., Abood, M. E. & Bourne, H. R. (1981): Cholera toxin can catalize ADP-ribosylation of cyoskeletal proteins. J. Cell. Biol., 91, 410−413.

20) Weisenberg, R. C. (1972): Microtubule formation in vitro in solutions containing low calcium concentrations. Science, 177, 1104−1105.

21) Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973): Microtubule assembly in the absence of added nucleotides. Proc. Natl. Acad. Sci. U. S. A, 70, 765−768.

22) Pruss, P. M., & Herschman, H. R. (1979): Cholera toxin stimulates division of 3T3 cells. J. Cel. Physiol., 98, 469−474.

23) Blume, A. J. & Levinson, S. (1979): Cholera toxin activation of neuroblastoma adenylate cyclase. Transmembrane Signaling, 675−684.

24) Laemmli, U. D. (1970): Cleavage of structural protein during the assembly of the head of bacteriophage T-4. Nature (Lond.), 227, 680−685.

25) Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265−275.

26) Nath, J. & Flavin, M. (1979): Tubulin tyrosylation in vivo and changes accompanying differentiation of cultured Neuroblastoma-Glioma Hybrid Cells. J. Biol. Chem., 254, 11505−11510.

27) Vigny, A., Huitorel, P., Henry, J. P., Pantaloni, D., (1980): Interaction of tyrosine hydroxylase with tubulin. Biochem. Biophy. Res. Commun., 92, 431−439.

28) Hayashi, M., Ohnishi, K. & Hayashi, K., (1980): Dense precipitate of brain tubulin with skeletal muscle myosin. J. Biochem., 87, 1347−1355.

29) Simooka, T., Hayashi, M. & Watanabe, Y. (1980): Tubulin-myosin interaction. Some properties of binding between tubulin and myosin. Biochem., 19, 4921−4926.

30) Feit, H. & Shay, J. W. (1980): The assembly of tubulin into membrananes. Biochem. Biophy. Res. Commun., 94, 324−331.

31) Hollenberg, M. D. & Cuatrecasas, P. (1973): Epidermal growth factor: Receptors in human fibroblasts and modulation of action by cholera toxin. Proc. Natl. Acad. Sci. U.S.A., 70, 2964−2968.

32) Hollenberg, M. D., Fishman, P. H., Bennett, V. & Cuatrecasas, P. (1974): Cholera toxin and cell growth: Role of membrane gangliosides. Proc. Natl. Acad. Sci. U.S.A., 71, 4224-4228.

33) Holmgren, J., Lindholm, L. & Lonnroth, L. (1974): Interaction of cholera toxin derivatives with lymphocytes. J. Exp. Med., 139, 801−819.

34) Derubertis, F. R., Zenser, T. V., Adler, W. H. & Hudson, T. (1974): Role of cyclic adenosine 3. 5-monophosphate in lymphocyte mitogenesis. J. Immun., 113, 151−161.

35) Vischer, T. L. & LoSpalluto, J. J. (1975): The differential effect of cholera toxin on the lymphocyte stimulation induced by various mitogens. Immunol., 29, 275−282.

36) Pawelek, J., Halaban, R. & Christie, G. (1975): Melanoma cells which require cyclic AMP for growth. Nature, 258, 539−540.

37) Raff, M. C., Hornby-Smith, A. & Brockes, J. P. (1978): Cyclic AMP as a mitogenic signal

for cultured rat Schwann cells. Nature, 273, 672−673.
38) Taylor-Papadimitoriou, J., Purkis, P. & Fentiman, I. S. (1980): Cholera toxin and analogues of cyclic AMP stimulate the growth of cultured human mammary epithelial cells. J. Cell. Physiol., 102, 317−321.
39) Davison, P. M. & Karasek, M. A. (1981): Human dermal microvascular endothelial cells *in vitro*: Effect of cyclic AMP on cellular morphology and proliferation rate. J. Cell. Physiol., 106, 253−258.
40) Green, H. (1978): Cyclic AMP in relation to proliferation of the epidermal cell: a new view. Cell., 15, 801−811.
41) Kuroki, T. (1981): Induction by cholera toxin of synchronous divisions *in vivo* in the epidermis resulting in hyperplasia. Proc. Natl. Aced. U.S.A., 78, 6958−6962.

コレラ毒素により ADP- リボース化したチューブリンの重合促進
江原雅彦・重野秀明（長崎大学熱帯医学研究所病原細菌学部門）

豚脳より精製したチューブリンへのコレラ毒素による影響を nucleotide regenerating system 下で生化学的，電顕的に観察した．コレラ毒素の ADP-ribosylation によりチューブリンは重合を促進し，かつ重合した微小管は安定した形を呈した．生体内でも同様な機序が考えられるとすれば，コレラ発症の機序として，チューブリンの ADP- リボース化から微小管への重合促進さらに下痢発症と云う事も想定される．今後，抗チューブリン抗体による酵素抗体法・螢光抗体法による実験コレラ症時の小腸上皮の所見が望まれる．

熱帯医学　第24巻　第3号　155−163頁，1982年9月